AWARD NUMBER: W81XWH-14-1-0557

TITLE: Making Aggressive Prostate Cancer Quiescent by Abrogating Cholesterol Esterification

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REPORT DATE: October 2016

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2016	Annual	30 Sep 2015 - 29 Sep 2016

4. TITLE AND SUBTITLE

Making Aggressive Prostate Cancer Quiescent by Abrogating Cholesterol Esterification

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-14-1-0557

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Purdue University
West Lafayette, IN 47907

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Our overall objective in the current application is to establish the viability of a new strategy of treating late stage PCa through therapeutic targeting of cholesterol metabolism in vivo, using combination of cutting edge spectroscopic imaging and other technologies, including biochemistry assays and preclinical testing. The innovation of this study is that it targets altered cholesterol metabolism, an understudied field of cancer research. Our central hypothesis is that abrogating cholesterol esterification events will result in an effective strategy for treating late stage PCa. This hypothesis will be tested by first validating the presence of altered cholesterol metabolism in human prostate cancer patient specimens. We will then evaluate the therapeutic benefit of CE depletion in appropriate animal models of PCa, and elucidate pathways linking cholesterol metabolism with cancer aggressiveness. An interdisciplinary research team has been assembled, with expertise in spectroscopic imaging & nanomedicine (Dr. J. X. Cheng, PI), biochemistry (Dr. X. Liu, co-PI), and prostate cancer biology (Dr. T. Ratliff, co-PI).

15. SUBJECT TERMS

None provided

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	14	19b. TELEPHONE NUMBER *(include area code)*

Lay Abstract:

Since the introduction of prostate specific antigen screening, prostate cancer has become the most widely diagnosed non-skin cancer in men in the United States (220,800 cases estimated in 2015). While often diagnosed in clinically localized stages, PCa remains the second leading cause of cancer-related mortality in American men with over 27,540 projected deaths in 2015. For men with advanced prostate cancer, androgen deprivation therapy in the form of bilateral orchiectomy or pharmacologic castration is an accepted standard therapy. Despite initial disease control, androgen deprivation therapy alone is non-curative and the subsequent development of castration-resistant prostate cancer (CRPC) occurs in the lifespan of almost all men who do not succumb to non-cancer deaths. For men with metastatic CRPC, docetaxel was approved in 2004 as the first-line cytotoxic chemotherapy owing to a modest increase in overall survival compared to mitoxantrone. Since 2010, there has been a tremendous increase in treatment options available for metastatic CRPC patients, including novel anti-androgen therapy with abiraterone and others. Nevertheless, the effectiveness of current therapies is palliative with an improvement in overall survival of 2-5 months compared to placebo. Therefore, a critical need exists to develop novel therapeutic strategies for advanced prostate cancer.

Cancer cells adopt metabolic pathways that differ from their normal counterparts by high rates of glycolysis and biosynthesis of essential macromolecules to fuel rapid growth. Among dysregulated metabolic pathways, altered lipid metabolism is increasingly recognized as a signature of cancer cells. Enabled by label-free coherent Raman scattering microscopy, our laboratory has performed the first quantitative analysis of lipogenesis at single cell level in human patient cancerous tissues. Our imaging data revealed an aberrant cholesterol ester accumulation in high-grade prostate cancer and metastases, but not in normal prostate or prostatitis. Cholesterol is an essential biomolecule that plays important roles in the maintenance of membrane structure, signal transduction, and provision of precursor to hormone synthesis. While cholesterol accumulation is known to be a hallmark of atherosclerosis, its exact role in cancer progression remains elusive. Our unexpected finding of cholesterol ester accumulation in advanced human prostate cancer triggered us to ask whether such cholesterol ester accumulation could become a potential target for prostate cancer treatment. Our pilot study has indeed showed that pharmacological inhibition of cholesterol ester accumulation significantly suppressed prostate cancer aggressiveness without affecting normal cell viability. Based on these appealing data, we hypothesize that abrogating cholesterol ester accumulation will result in an effective strategy for treating advanced prostate cancer. This hypothesis will be tested through two specific aims. First, we will develop a clinically viable strategy of cholesterol depletion and evaluate its therapeutic effect on tumor growth in appropriate animal models of prostate cancer. Second, to understand how such treatment strategy benefits prostate cancer, we will elucidate the mechanism by which cholesterol ester accumulation contributes to prostate cancer aggressiveness.

At the completion of this project, it is our expectation that we will have provided strong evidences to support the concept that inhibition of cholesterol ester accumulation is a viable and potentially attractive therapeutic intervention strategy to treat advanced prostate cancer. Notably, several small molecule inhibitors of cholesterol accumulation, e.g. avasimibe, have gone through clinical trials to treat atherosclerosis but failed due to the lack of effectiveness. Our proposed study will demonstrate a novel use of existing drugs to treat advanced prostate cancer, and it is anticipated that preclinical studies and/or clinical trials will follow shortly after the completion of this project. Ultimately, the adoption of such strategy will substantially improve the clinical outcome for metastatic prostate cancer patients that are resistant to hormone therapy. Our deeper mechanistic study will contribute to the understanding of dysregulated cholesterol metabolism in advanced prostate cancer, which in turn provides the biological foundation of targeting cholesterol accumulation for treatment of metastatic prostate cancer.

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1. INTRODUCTION:

Our *overall objective in the current application* is to establish the viability of a new strategy of treating late stage PCa through therapeutic targeting of cholesterol metabolism *in vivo*, using combination of cutting edge spectroscopic imaging and other technologies, including biochemistry assays and preclinical testing. *The innovation of this study* is that it targets altered cholesterol metabolism, an understudied field of cancer research. Our *central hypothesis* is that abrogating cholesterol esterification will result in an effective strategy for treating late stage PCa. This hypothesis will be tested by first validating the presence of altered cholesterol metabolism in human prostate cancer patient specimens. We will then evaluate the therapeutic benefit of CE depletion in appropriate animal models of PCa, and elucidate pathways linking cholesterol metabolism with cancer aggressiveness. An interdisciplinary research team has been assembled, with expertise in spectroscopic imaging & nanomedicine (Dr. J. X. Cheng, PI), biochemistry (Dr. X. Liu, co-PI), and prostate cancer biology (Dr. T. Ratliff, co-PI).

2. KEYWORDS:

Prostate cancer, lipid droplet, metabolism, cholesterol, cholesteryl ester, Raman spectroscopy

3. ACCOMPLISHMENTS:

a. What were the major goals of the project?

The two major goals of this project are (1) Develop a clinically viable cholesteryl ester depletion strategy to suppress the proliferation of late-stage prostate cancer *in vivo*; (2) Determine the relative contribution of altered cholesterol metabolism to prostate cancer aggressiveness.

b. What was accomplished under these goals?

During year 2 of this project, we have accomplished **task 3**: Determine the consequence of avasimibe administration on arachidonic acid and cholesteryl ester levels in prostate cancer cells. Furthermore, we have accomplished **task 4**: Establish a solid correlation between cholesteryl ester accumulation / depletion and the potential of tumor cell migration *in vitro*. Meanwhile, we have accomplished part of **task 5**: Elucidate the pathway linking CE accumulation and PCa cell aggressiveness. Detailed results are shown below.

Task 3: Determine the consequence of avasimibe administration on arachidonic acid (AA) and cholesteryl ester levels in prostate cancer cells.

Our liquid chromatography-mass spectrometry analysis of PC-3 cell lysates revealed that ACAT inhibition by avasimibe or ACAT-1 knockdown by shRNA significantly reduced the level of AA in PC-3 cells **(Figure 3.1)**

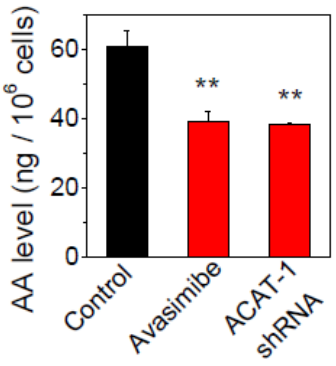

Figure 3.1. AA levels in PC-3 cells treated with avasimibe or ACAT-1 shRNA (n = 3). Avasimibe treatment: 7.5 μM, 2 day; ACAT-1 shRNA: 3 day transfection.

We further studied the lipid levels in the PC3 tumor model. SRL imaging showed a large amount of lipid accumulation in the tumor tissues (**Figure 3.2A**). Avasimin treatment distinctly reduced the amount of LDs for the PC3 tumors (**Figure 3.2A**). Raman spectra from LDs in the tumor tissues demonstrated the cholesterol ring vibration band at 702 cm^{-1} (**Figure 3.2B**). The CE level (25±5 %) in LDs of PC3 tumor tissue was smaller than that of cultured PC3 cells (74±15 %) (**Figure 3.2C**). The increase (more than 30 %) in free cholesterol levels for the tumor treated with avasimin was also determined (**Figure 3.2D**).

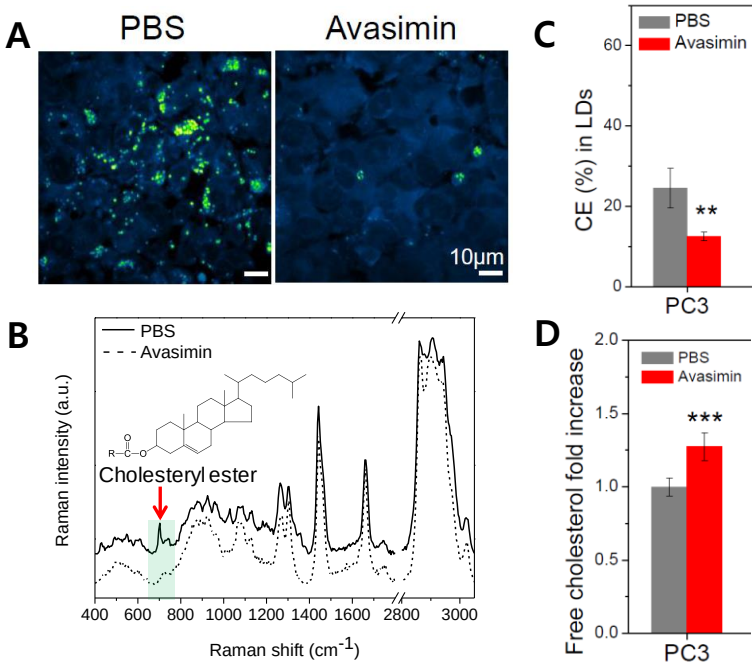

Figure 3.2. Pharmacodynamics of avasimibe treatment in a mouse model of prostate cancer.

Task 4: Establish a solid correlation between cholesteryl ester accumulation / depletion and the potential of tumor cell migration *in vitro*.

In order to evaluate the potential of tumor cell migration, we performed standard transwell assays in prostate cancer cells. Cholesteryl ester depletion by avasimibe, Sandoz, or ACAT-1 knockdown using shRNA suppressed migration and invasion capabilities of PC-3 cells (**Figure 4.1**). On the other hand, when the cholesteryl ester accumulation is promoted by PTEN

knockdown using shRNA in DU145 cells, we observed greater capabilities of migration and invasion compared to the cholesteryl ester-poor PTEN wild-type DU145 cells (**Figure 4.2**).

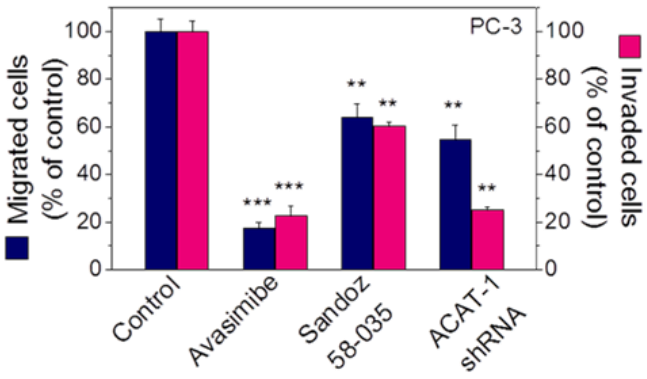

Figure 4.1. Quantification of migrated and invaded PC-3 cells pre-treated with avasimibe, Sandoz 58-035, or ACAT-1 shRNA (n = 3). Avasimibe treatment: 5 µM, 2 days; Sandoz 58-035 treatment: 10 µM, 2 days; ACAT-1 shRNA: 2 day transfection.

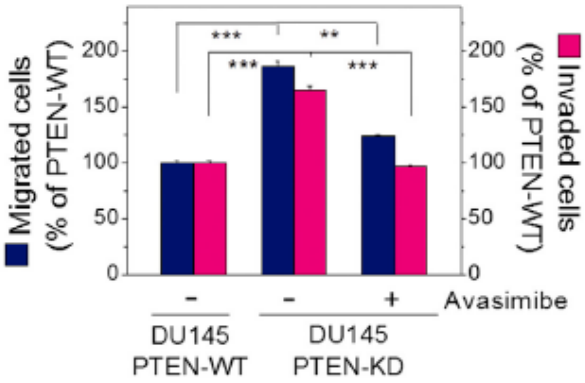

Figure 4.2. Quantification of migrated and invaded DU145 cells with PTEN wild-type or PTEN knockdown using shRNA (n = 3). Avasimibe treatment: 5 µM for 1 day.

We further compared migration/invasion capabilities of two LNCaP cell lines, LNCaP-LP (low passage) and LNCaP-HP (high passage). LNCaP-HP cells were derived upon continuous passage from LNCaP-LP until the passage number was over 60. Compared to cholesteryl ester poor LNCaP-LP cells, cholesteryl ester-rich LNCaP-HP cells showed greater migration and invasion capabilities (**Figure 4.3**).

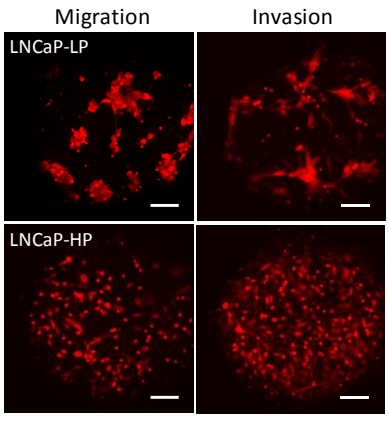

Figure 4.3. Representative images of migration and invasion of LNCaP-LP and LNCaP-HP cells. Scale bar: 50 µm.

To summarize, we established a solid correlation between cholesteryl ester accumulation / depletion and the potential of tumor cell migration in vitro. Cholesteryl ester accumulation promotes prostate cancer migration and invasion potentials in multiple prostate cancer cell lines. When cholesteryl ester is depleted either by ACAT-1 inhibitors or genetic manipulations, cancer cells showed reduced migration and invasion capabilities (**Table 1**).

Table 1. Effect of cholesteryl ester accumulation on prostate cancer cell migration potential.

	PC-3	PC-3 + ACAT-1 inhibitors	PC-3 + ACAT-1 KD	DU145 PTEN WT	DU145 PTEN KD	LNCaP-LP	LNCaP-HP
CE level	High	Low	Low	Low	High	Low	High
Migration / invasion potential	High	Low	Low	Low	High	Low	High

Task 5: Elucidate the pathway linking CE accumulation and PCa cell aggressiveness.

We performed gene expression analysis using RT^2 Profiler PCR array, in which we looked at expression levels of 84 human prostate cancer related genes after inhibiting cholesteryl ester storage in PC3 cells. Our results revealed that multiple negative regulators of metastasis were upregulated after avasimibe treatment in PC3 cells (**Figure 5.1**).

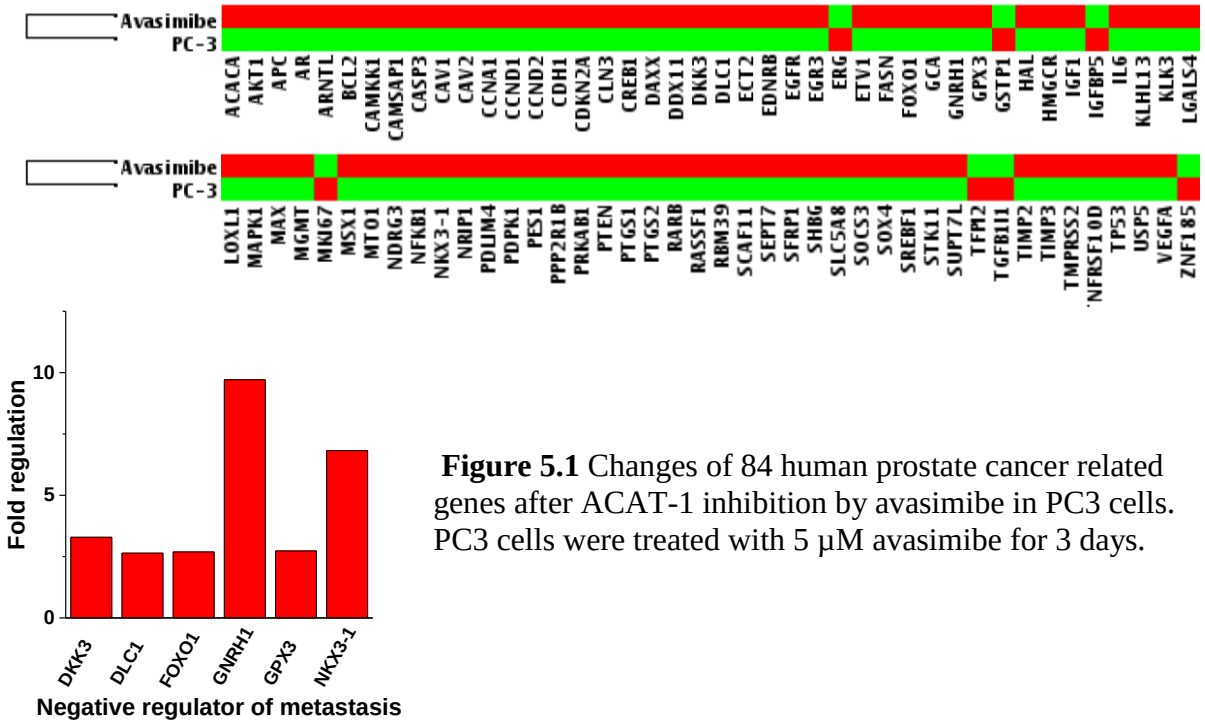

Figure 5.1 Changes of 84 human prostate cancer related genes after ACAT-1 inhibition by avasimibe in PC3 cells. PC3 cells were treated with 5 μM avasimibe for 3 days.

Among these upregulated genes, DKK3 is a negative regulator of Wnt/β-catenin pathway, a major pathway associated with metastasis in prostate cancer. Immunoblotting of β-catenin further confirmed that ACAT-1 inhibition by avasimibe or ACAT-1 knockdown by shRNA significantly downregulated Wnt/β-catenin pathway (**Figure 5.2**). As an independent evidence, immunofluorescent staining of β-catenin showed decrease in the nuclear localized β-catenin after avasimibe treatment, indicating inactivation of Wnt/β-catenin pathway (**Figure 5.3**).

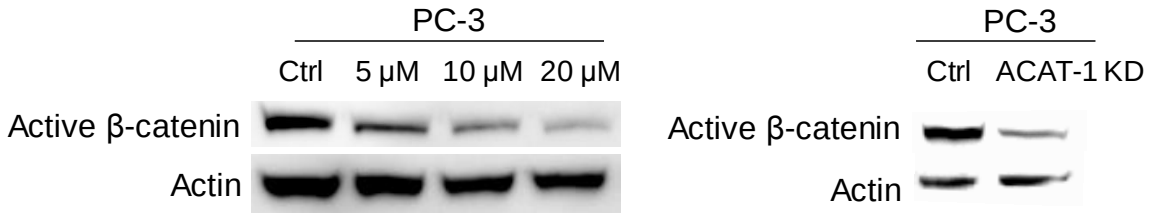

Figure 5.2. Immunoblotting of β-catenin in PC-3 cells with avasimibe or ACAT-1 shRNA. Avasimibe were treated with the indicated concentration for 3 days. ACAT-1 knockdown PC-3 cell line with stable ACAT-1 knockdown was generated by transducing with ACAT-1 shRNA containing lentivirus.

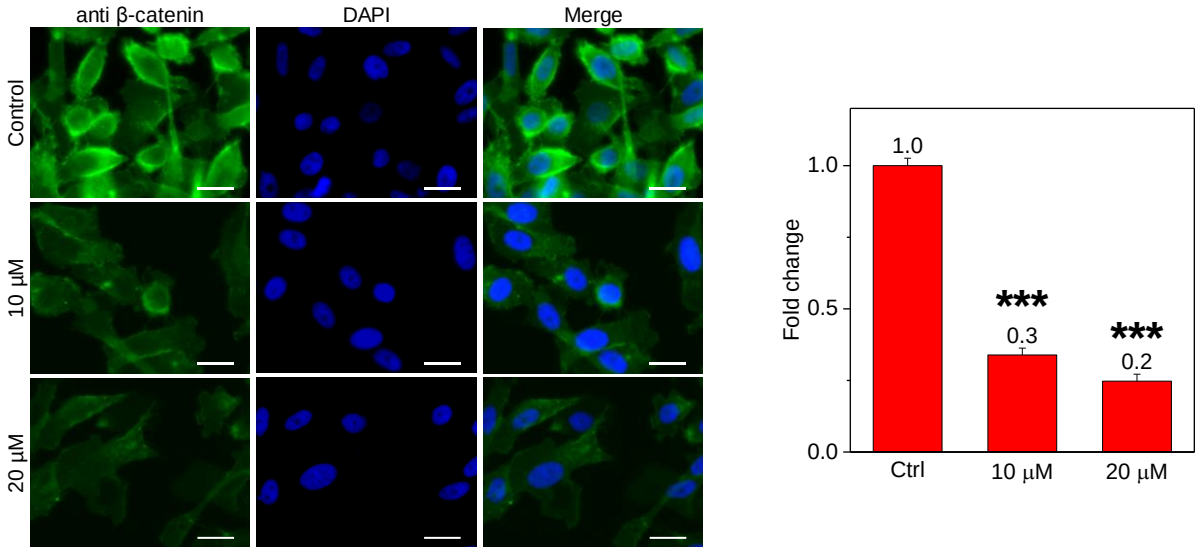

Figure 5.3. Immunofluorescent staining of β-catenin in PC-3 cells with avasimibe. Avasimibe were treated with the indicated concentration for 3 days.

We further studied whether downregulation of β-catenin by inhibition of ACAT-1 is found in other cholesteryl ester-rich prostate cancer cells, we measured β-catenin levels in LNCaP-HP cells with avasimibe treatment. From immunoblotting of β-catenin, we found that β-catenin pathway is also downregulated upon avasimibe treatment (**Figure 5.4**).

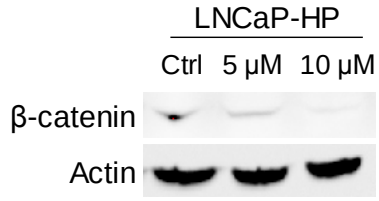

Figure 5.4. Immunoblotting of β-catenin in LNCaP-HP cells with avasimibe. Avasimibe were treated with the indicated concentration for 3 days.

c. **What opportunities for training and professional development has the project provided?**

In total, two postdoctoral fellows (Junjie Li, Jack Li), three PhD students (Seung Young Lee, Hyeon Jeong Lee, Renee E Wenig), and two undergraduate students (Jien Nee Tai, Rui Liu) worked on this project. Seung Young Lee has graduate and is now a postdoc at Boston University. Junjie Li co-founded a company Resarci Therapeutic LLC to repurpose avasimibe for cancer treatment.

d. **How were the results disseminated to communities of interest?**

The results were disseminated to communities of interest through a few invited presentations:

09-09-2016, "Lipid metabolism: from single cell biology to in vivo diagnosis", Big Ten Cancer Research Consortium Summit, Indianapolis, IN.

06-29-2016, "Molecular spectroscopic imaging towards precision medicine", Cancer Moonshot, Purdue University.

e. **What do you plan to do during the next reporting period to accomplish the goals?**

The data we obtained in year 2 indicate that avasimibe treatment suppresses metastasis in prostate cancer through down-regulating the Wnt/beta-catenin pathway. During the next funding period (year 3), we will continue to elucidate this pathway using biochemistry and molecular biology tools, via collaboration with Xiaoqi Liu, a biochemistry and co-investigator in this DoD grant. In parallel, we will use the established orthotopic prostate cancer model to evaluate the impact of avasimibe treatment on prostate cancer metastasis rate.

4. **IMPACT:**

a. **What was the impact on the development of the principal discipline(s) of the project?**

Nothing to report.

b. **What was the impact on other disciplines?**

Nothing to report.

c. **What was the impact on technology transfer?**

US9164084 B2 “A method for determining aggressiveness of a cancer and treatment thereof” Filed 10/20/2015. This IP is based on our finding of cholesterol ester storage in aggressive cancer.

d. **What was the impact on society beyond science and technology?**

Nothing to report.

5. **CHANGES/PROBLEMS:**

a. **Changes in approach and reasons for change**

Nothing to report.

b. **Actual or anticipated problems or delays and actions or plans to resolve them**

We have some delay in developing the orthotopic prostate cancer model at Purdue. This delay might impact our progress in year 3. If needed, we would like to ask 1-year no cost extension. Thanks for understanding.

c. **Changes that had a significant impact on expenditures**

Nothing to report.

d. **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

Nothing to report.

e. **Significant changes in use or care of human subjects**

Nothing to report.

f. **Significant changes in use or care of vertebrate animals.**

Nothing to report.

g. **Significant changes in use of biohazards and/or select agents**

Nothing to report.

6. **PRODUCTS:**

a. **Publications, conference papers, and presentations**
Report only the major publication(s) resulting from the work under this award.

i. **Journal publications.**

Seung-Young Lee, Junjie Li, Jien-Nee Tai, Timothy L. Ratliff, Kinam Park, Ji-Xin Cheng*, "Avasimibe encapsulated in human serum albumin blocks cholesterol esterification for selective cancer treatment", ACS Nano, 2015, 3: 2420-2432.

Acknowledgement of DoD support (yes).

ii. **Books or other non-periodical, one-time publications.**

Nothing to report.

iii. **Other publications, conference papers, and presentations.**

Nothing to report.

b. **Website(s) or other Internet site(s)**
Nothing to report.

c. **Technologies or techniques**
Nothing to report.

d. **Inventions, patent applications, and/or licenses**

Based on the albumin formulation of avasimibe, a non-provisional patent was filed through Purdue University, filing date: Sept 10, 2015, application No. 14/850,941

"Cholesteryl Ester-Depleting Nanomedicine for Nontoxic Cancer Chemotherapy", PRF 66947

e. **Other Products**
Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

a. **What individuals have worked on the project?**

Name: Professor Ji-Xin Cheng
Project role: PI
Research Identifier: NA
Nearest person month worked: 1 month
Contribution to project: Dr. Cheng guided the entire project. He had weekly meetings with the students and organized bio-monthly meetings for the entire team.

Name: Professor Xiaoqi Liu
Project role: co-investigator
Research Identifier: NA
Nearest person month worked: 0.5 month
Contribution to project: Experimental design during lunch meetings and regular bio-monthly meetings.

Name: Professor Tim Ratliff
Project role: co-investigator
Research Identifier: NA
Nearest person month worked: 0.5 month
Contribution to project: Experimental design during lunch meetings and regular bio-monthly meetings.

Name: Hyeon Jeong Lee
Project role: graduate student
Research Identifier: NA
Nearest person month worked: 8 months
Contribution to project: Ms. Lee obtained data showing the avasimibe treatment effected reduced the rate of tumor migration and invasion in vitro. She further found that avasimibe treatment impacted the activity of the Wnt/beta-catenin pathway.

Name: Renee Wenig
Project role: graduate student
Research Identifier: NA
Nearest person month worked: 1 month
Contribution to project: Ms. Wenig helped Ms. Lee in the study of the Wnt/beta-catenin pathway.

Name: Rui Liu

Project role: undergraduate student
Research Identifier: NA
Nearest person month worked: 1 month
Contribution to project: Ms. Liu helped Ms. Lee in the biochemistry experiments. She also maintained the cell culture.

Name: Dr. Junjie Li
Project role: postdoctoral fellow
Research Identifier: NA
Nearest person month worked: 1 month
Contribution to project: Mr. Li helped Ms. Lee in performing the tumor migration assay.

Name: Dr. Jack Li
Project role: postdoctoral fellow
Research Identifier: NA
Nearest person month worked: 1 month
Contribution to project: Dr. Li helped Ms. Lee in performing the western blotting assays and immunofluorescence imaging.

b. **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Nothing to report.

c. **What other organizations were involved as partners?**

Nothing to report.

8. **SPECIAL REPORTING REQUIREMENTS**

a. **COLLABORATIVE AWARDS: NA**

b. **QUAD CHARTS: NA**

9. **APPENDICES:** NA